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N-Acetyl lysyl-tRNA synthetases evolved by a CcdB-based selection possess *N*-acetyl lysine specificity in vitro and in vivo

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ABSTRACT

Posttranslational modifications play a crucial role in modulating protein structure and function. Genetic incorporation of unnatural amino acids into a specific site of a protein facilitates the systematic study of protein modifications including acetylation. We here report the directed evolution of pyrrolysyl-tRNA synthetase (PylRS) from *Methanosarcina mazei* to create *N*-acetyl lysyl-tRNA synthetases (AcKRSs) using a new selection system based on the killing activity of the toxic *ccdB* gene product. The amino acid specificity of these and of published [1,2] AckRSs was tested in vitro and in vivo, and the enzyme-kinetic properties of the AckRSs were evaluated for the first time.

1. Introduction

Posttranslational modifications (PTM) markedly expand the natural repertoire of proteins by enlarging the diversity of functional groups beyond those of naturally occurring 20 amino acids. A wide range of cellular processes are governed by various types of PTM, including phosphorylation, glycosylation, and acetylation [3]. Thus, elucidation of the molecular mechanisms and cellular functions of PTM is fundamental to understanding the complicated biological system. For a systematic study of PTM, preparation of a homogenously modified protein is essential. Many methods including selective chemical modifications and native chemical ligation have been developed, but these methods suffer from a lack of generality [4]. They require tedious chemical synthesis and ligation, and they can only be applied to N- and C-terminal modifications. Hence, production of recombinant proteins with defined and homogenous modification still remains a challenge.

Cotranslational insertion of an unnatural amino acid into a specific site of a protein is a powerful tool for investigating protein structure and function relationship, probing protein function in biological processes, and manipulating proteins with tailor-made functionalities. To date we know of a large number of unnatural amino acids that have been genetically incorporated into proteins by pairs of an engineered aminoacyl-tRNA synthetase (aaRS) and its cognate suppressor tRNA; these pairs are orthogonal to the host tRNAs and aaRSs [5–7]. *N*-Acetyl lysine has been site-specifically incorporated into proteins through enzymes derived by evolution of PylRS [1,2]. Recently the molecular evolution of elongation factor (EF-Tu) [8] has been used for the successful incorporation of phosphoserine into proteins. The genetic incorporation approach has proved effective at producing homogenously modified proteins, but it often produces relatively low levels of recombinant proteins (1–2 mg/l) in part due to low in vivo charging activity of the evolved aaRSs.

Directed evolution experiments rely on two factors, generation of diverse population and the use of the proper selection system. In particular, the development of an efficient selection method is often considered the most crucial step in these experiments, as illustrated in the phrase "you get what you select for" [9]. Most aaRS evolution experiments have been based on CAT and barnase based selection [1,5,10]. Since selection method significantly affects the outcome of directed evolution of enzymes, we reasoned that different selection methods might generate different pools of positive variants in the aaRS evolution experiments. Therefore, the development of a distinct selection system might expedite the evolution of aaRS with desirable in vivo charging activity, which might lead to high yield production of recombinant proteins with defined modifications. In this report, we have constructed a new negative selection system based on the killing activity of the lethal *ccdB* gene and applied this system to the evolution of AcKRS from *Methanosarcina*

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mazei PylRS for genetic incorporation of *N*-acetyl lysine (AcK). Catalytic properties of the evolved AckRSs were examined using both in vivo charging and suppression experiments and in vitro ATP-PPi exchange assay.

2. Materials and methods

2.1. General

N-Acetyl lysine (AcK) was purchased from Sigma. Oligonucleotide synthesis and DNA sequencing were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. All restriction enzymes and DNA polymerase were purchased from New England Biolabs.

2.2. Plasmids construction

To generate a new negative selection system, we initially constructed pCB plasmid carrying *pylT* and *ccdB* gene with amber stop codon on different sites using pTECH plasmid [11] and pZErO-2 vector (Invitrogen). We then generated pAraCB2 plasmid for tight regulation of the *ccdB* gene by employing ara promoter and rrnB terminator. More details are listed in Supplementary data.

2.3. MmPylRS mutant library construction and selection for AcK-specific MmPylRS variants

For mutant library construction, six residues (L301, L305, Y306, L309, C348, and W417) of *M. mazei* PylRS were randomly mutagenized by using eight primers and overlap extension PCR method as described before [12]. The MmPylRS mutant library was screened through a serious of CAT and CcdB based selection methods. For the first positive selection, 50 ng of pKTS-MmPylS library plasmid was introduced into 120 μl electrocompetent *Escherichia coli* TOP10 (4.2×10^9 cells) with positive selection plasmid pCAT-pylT that has a mutant *cat* gene with an amber stop codon at position 112 and $tRNA^{Pyl}$. The transformants were recovered in 3 ml SOC at 37 °C for 2 h. It was transferred and cultivated in 100 ml LB-TK (containing 10 μg/ml Tet and 25 μg/ml Km) for 16 h at 37 °C. 400 μl of the overnight culture was inoculated into 20 ml fresh LB-TKack (LB-TK containing 1 mM AcK). After growing at 37 °C for 4 h, 500 μl was plated on ten LB-TKCack (LB-TK containing 50 μg/ml Cm and 1 mM AcK) plates. The plates were incubated at 37 °C for 42 h, all the colonies growing on the plates were scraped and resuspended in 25 ml LB-TKack. After incubation for 5 h at 37 °C, plasmids were extracted and library plasmids were purified from pCAT-pylT plasmids. For negative selection, 10 ng of pKTS-MmPylS plasmids from the positive selection was transformed into 40 μl electrocompetent *E. coli* TOP10 (1.2×10^9 cells) with negative selection plasmid pAraCB2-pylT (see Supplementary data). The transformants were recovered in 2 ml SOC at 37 °C for 3 h, and then 60 μl was plated on six LB-CKara (containing 34 μg/ml Cm, 25 μg/ml Km and 0.2% arabinose) plates. After incubation at 37 °C for 24 h, the surviving colonies were scraped and pKTS-MmPylS plasmids were isolated and used for additional positive selection. Forty eight colonies were selected at this stage and the suppression activity of individual colony was checked. Eleven clones were found to show AcK-dependent growth on LB-TKCacK plate.

3. Results and discussion

3.1. Design and construction of CcdB negative selection system

In *E. coli*, the ccd system (*ccdA* and *ccdB*) of the F plasmid is responsible for the plasmid's high stability by post-segregational killing of plasmid-free cells [13]. CcdB is a toxin targeting the essential DNA gyrase while CcdA functions as an antitoxin. To construct a negative selection system using the *ccdB* gene, we searched for permissive site(s) allowing efficient termination of an amber stop codon and full restoration of the killing activity when the stop codon is suppressed. We focused on the specific regions of the CcdB protein (10–13 and 44–47 residues) because they are located on the outside loop regions and not involved in dimerization or interaction with the gyrase (Supplementary Fig. S1). We initially constructed four kinds of pCB plasmids, pCB-R10-pylT, pCB-E11-pylT, pCB-S12-pylT, and pCB-R13-pylT carrying *pylT* and the *ccdB* gene (under the control of *lac* promoter) with stop codon at position Arg10, Glu11, Ser12, or Arg13 (Supplementary Fig. S2A). $tRNA^{Pyl}$ and its anticodon mutants (opal and ochre suppressors) are functional and orthogonal to *E. coli* aaRSs [14,15]. So, the efficiency of translation termination of the mutant *ccdB* gene in the presence of *pylT* is mainly dependent upon the nucleotide context of the stop codon [16]. Analysis of the transformants of each plasmid revealed that pCB-R13-pylT showed the lowest non-specific readthrough level but it still showed a retarded growth (Supplementary Fig. S2B). We next introduced second amber stop codon at position 44 to construct pCB-R13D44-pylT. A growth phenotype of the transformants with pCB-R13D44-pylT was indistinguishable from that of the cells devoid of the *ccdB* gene (Supplementary Fig. S2B). When *pylT* gene was replaced with a suppressor $tRNA^{Gln}$ (*supE*), the transformants of pCB-R13D44-supE could not grow. We then constructed pAraCB2-pylT plasmid carrying *ccdB* gene with double stop codons under the control of *ara* promoter for a tight and rapid regulation (Supplementary Fig. S2C). To further verify the efficiency of CcdB selection system, we examined whether the selection system worked for an orthogonal *M. mazei* PylRS and its cognate tRNA pair (MmPylRS/$tRNA^{Pyl}$). The transformants with pAraCB2-pylT/pKTS and those with pAraCB2-pylT/pKTS-MmPylRS showed normal growth pattern with or without arabinose, suggesting negligible level of background *ccdB* gene expression (Supplementary Fig. S2D). On the other hand, the transformants with pAraCB2-pylT/pKTS-MmPylRS could not survive in the presence of *N*-cyclopentyloxycarbonyl-L-lysine (Cyc), a pyrrolysine analog for MmPylRS [17]. These results clearly showed that the CcdB selection system worked well for an orthogonal MmPylRS/$tRNA^{Pyl}$ pair and it can be applied for the evolution of aaRS/tRNA pair for the incorporation of unnatural amino acids with diverse functionalities.

3.2. Evolution of PylRS using CcdB selection system

To test the idea that a distinct selection system might generate somewhat different pools of positive variants of aaRS, we set out to evolve MmPylRS/$tRNA^{Pyl}$ pair to incorporate AcK by using CAT and CcdB based selection system. We first designed and generated a library of MmPylRS variants, where six residues (L301, L305, Y306, L309, C348, and W417) forming pyrrole ring binding pocket in the active site were randomized based on MmPylRS and pyrrolysine complex structure [18,19]. A library of 1.5×10^8 MmPylRS variants was introduced into *E. coli* TOP10 carrying $tRNA^{Pyl}$ and subjected to a series of positive and negative selections as described in the Methods. After second positive selection, 11 clones were isolated. Sequence analysis revealed that they were two different clones with four mutations; L301M, Y306L, L309A, C348F for MmAcKRS1, and L301M, Y306L, C348S and an undesigned mutation A315V for MmAcKRS2 (Table 1). Sequence comparison revealed that the mutation profile of the evolved MmAcKRSs differed from that of MbAcKRSs, although equivalent amino acid positions were randomized (Table 1). While barnase system produced MbPylRS variants with the same mutations on three residues L270I, L274A, C313F [1,2], MmAcKRS mutants selected by using

Table 1
Sequence comparison of MmAcKRSs and MbAcKRSs.

MmPylRS	L301	L305	Y306	L309	C348	W417	Reference
MmAcKRS1	**M**	**L**	**L**	A	F	**W**	This work
MmAcKRS2[a]	**M**	**L**	**L**	L	S	**W**	This work
MbAcKRS1	V	*I*	F	*A*	*F*	*W*	[1]
MbAcKRS2	L	*I*	L	*A*	*F*	*W*	[1]
MbAcKRS3	M	*I*	F	*A*	*F*	*W*	[2]
MbPylRS	L266	L270	Y271	L274	C313	W383	

Consensus sequences are shown in bold or italic type.
[a] This variant has an additional mutation A315V.

CcdB system have consensus sequences on different residues, L301M, L305, and Y306L. These results suggest that different selection systems may produce different pools of mutant clones possibly due to the differences in action mechanism or degree of stringency in the selection. However, other explanations are also possible. Interestingly, W417 in MmPylRS or W383 in MbPylRS was not changed in all AcKRSs, suggesting that W417 in MmPylRS may be crucial for the recognition of wide range of lysine analogs [19,20].

3.3. Characterization of evolved AcKRSs

In vivo aminoacylation activity of the evolved AcKRSs was analyzed by acid urea gel electrophoresis. The level of aminoacylation was visualized by Northern hybridization (Fig. 1). While wild type MmPylRS had a negligible charging activity for AcK, the evolved synthetases (MmAcKRS1 and MmAcKRS2) were able to acylate only AcK (not natural amino acids) onto $tRNA^{Pyl}$, generating AcK-$tRNA^{Pyl}$. The aminoacylation products were deacylated when treated with alkali (200 mM Tris–HCl, pH 9.5). To confirm these data, the in vitro activity of a truncated AcKRS version was measured in the ATP-PP_i exchange reaction as described earlier ([21]; see Supplementary data). Since there is a lack of studies reporting enzyme-kinetic properties of evolved orthogonal tRNA synthetases we perceived it essential that in vitro characterization of the PylRS variants from this study (MmAcKRS1 and MmAcKRS2) and from the original report (MbAcKRS3) [2] should be performed. PylRS is a fairly insoluble protein; thus the crystal structures and most of the original biochemical data of this enzyme were performed with a truncated PylRS version devoid of the variable N-terminal domain of the enzyme [18,19,22]. The truncated catalytic domain of PylRS was reported to aminoacylate $tRNA^{Pyl}$ with pyrrolysine with slightly lower efficiency than the full length PylRS [19]. We attempted to determine the kinetic constants for AcK, lysine, and the pyrrolysine analog *N*-cyclopentyloxycarbonyl-L-lysine (Cyc) in the ATP-PP_i exchange reaction (Table 2). The initial experiments revealed that Lys cannot be activated by any of the three enzymes, while Cyc is slightly activated by MmAcKRS2 (but the activity is too low to calculate any constants), but not by the two other enzymes. The evolved MmAcKRS1 and MmAckRS2 have good substrate specificity in recognizing AcK. Both MmAckRSs do not show any detectable activity toward lysine, which is in good agreement with in vivo charging results (Fig 1). In addition, the constants for a previously reported [2] enzyme, MbAcKRS3 (see Table 1), were also determined. This enzyme has an in vitro activity

Table 2
Kinetic parameters of AckRSs determined by ATP-PPi exchange assays.

Enzyme	Lysine		*N*-Acetyl lysine	
	K_m (mM)	K_{cat} (s^{-1})	K_m (mM)	K_{cat} (s^{-1})
MmAcKRS1	ND	ND	35.3 ± 10.9	0.0323 ± 0.00606
MmAcKRS2	ND	ND	7.80 ± 2.14	0.00731 ± 0.000767
MbAcKRS3	ND	ND	22.3 ± 4.35	0.0341 ± 0.00297

ND, the kinetic constants could be not be calculated because the signal was undetectably low.

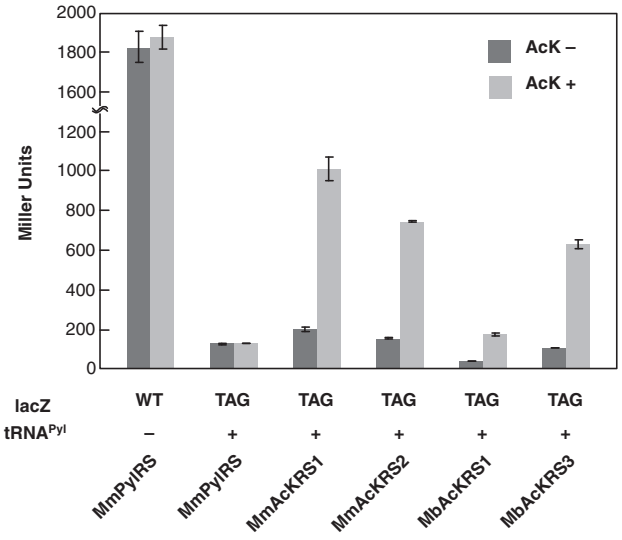

Fig. 2. In vivo β-galactosidase assay of the selected AcKRSs in the presence or absence of AcK.

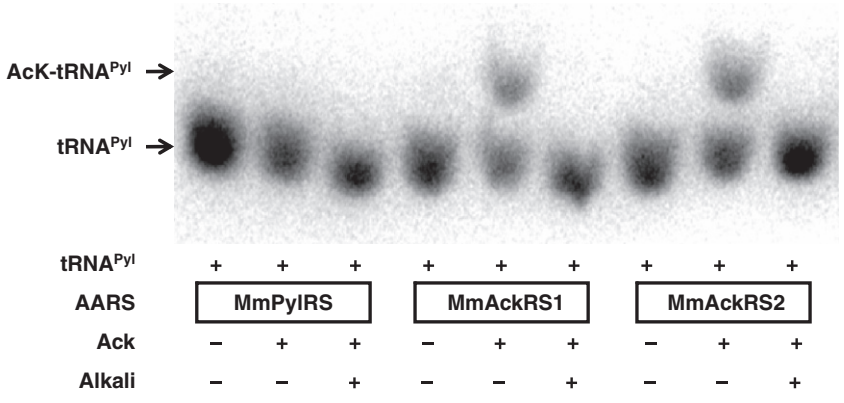

Fig. 1. In vivo aminoacylation of $tRNA^{Pyl}$ with AcK by AcKRS1 and AcKRS2. Total tRNA was extracted from *E. coli* TOP10 co-transformed with *pylT* and the gene for PylRS, AcKRS1, or AcKRS2 after grown in the presence or absence of 5 mM AcK. The samples were loaded onto an acid urea gel, transferred to a nitrocellulose membrane, and hybridized with a $tRNA^{Pyl}$-specific oligonucleotide probe. The positions of $tRNA^{Pyl}$ and AcK-$tRNA^{Pyl}$ are indicated.

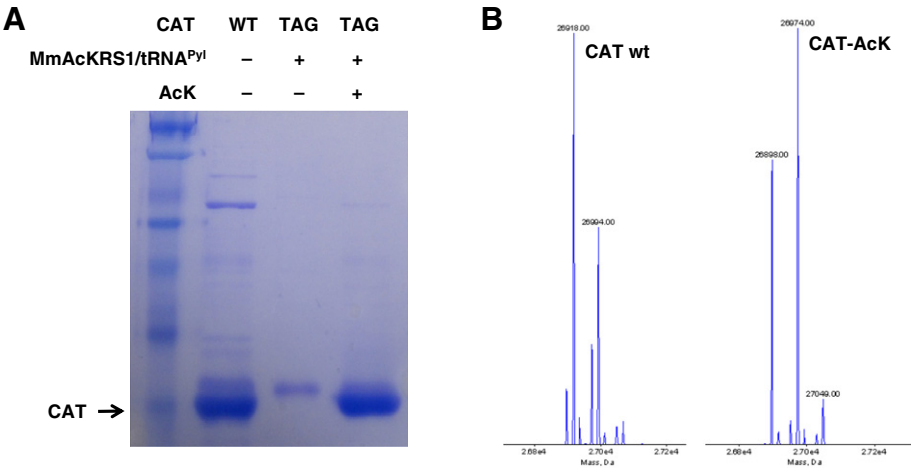

Fig. 3. Expression and analysis of a AcK-incorporated reporter protein. (A) Purified CAT wild type and CAT-AcK mutant resolved on 10% SDS–PAGE. (B) ESI-MS analysis of the purified CAT wild type (left) and CAT-AcK mutant (right).

similar to MmAcKRS1 and MmAcKRS2, and same specificity as MmAcKRS1. Regarding k_{cat}, the evolved AcKRSs (MmAckRS1 and MbAckRS3) showed a comparable value to the wild-type PylRS (around 2 min^{-1} for AckRSs and 6 min^{-1} for PylRS [23]). However, their K_m values were much higher, which may be due to imperfect AcK binding. This is not unexpected, since the change in only five amino acid residues of PylRS obviously is not generating an ideal amino acid binding site for AcK in the newly evolved synthetases.

To further examine the properties of the selected MmAcKRSs, we performed β-galactosidase assay where the suppression of *lacZ* gene with an amber stop codon at position 3 (Met) was measured. MmAcKRS1 and MmAcKRS2 both showed high levels of suppression activity only in the presence of AcK, proving that they were evolved to become AcK-specific aminoacyl-tRNA synthetases (Fig. 2). In particular, in vivo suppression activity of MmAcKRS1 was around ten and two times higher than that of previously reported AcKRSs, MbAcKRS1 and MbAcKRS3, respectively [1,2]. This in vivo assay produced different results than our in vitro ATP-PPi experiments, where the MmAcKRS1 and MmAcKRS2 showed a little lower catalytic efficiency than MbAcKRS3 (Table 2). One of the reasons may be that the truncated AcKRSs were used in ATP-PPi exchange analysis due to the limited solubility of PylRS [18,19]. The truncated AcKRSs may not fully represent in vivo performance of intact enzymes, since the in vivo approach measures translation as a whole while the in vitro approach measures only the behavior of a pure enzyme and a pure substrate.

To demonstrate that AcK can be incorporated into proteins with high fidelity and yield by the evolved MmAckRSs, we transformed *E. coli* TOP10 with pKTS-MmAcKRS1 and pCAT-pylT encoding *cat* gene with an amber stop codon (112Asp) and C-terminal hexahistidine tag. The transformants were cultured in LB medium supplemented with appropriate antibiotics and 20 mM nicotinamide and induced for protein expression with 0.5 mM IPTG for 12 h at 30 °C in the presence or absence of 2 mM AcK. The cells produced full length CAT recombinant proteins only when incubated with AcK with a yield of 6 mg/l of culture (Fig. 3A), which is several times higher than the yields by previously reported MbAcKRSs that lie in the range of 1–2 mg/l [1,2]. This agrees well with in vivo suppression data. To confirm the incorporation of AcK, purified his-tagged wild type CAT and mutant CAT-AcK were analyzed by electrospray ionization mass spectrometry (ESI-MS). Mass spectrum of CAT and CAT-AcK revealed that cysteine side chains are modified by β-mercaptoethanol (β-ME), leading to a 76 Da mass increase. In vitro chemical modification β-ME during protein purification has been reported [24]. Wild type CAT generated two main peaks (26918 and 26994 Da), representing the masses of CAT with single and double β-ME adduct (expected mass 26918 and 26994 Da, respectively) (Fig. 3B left). ESI-MS analysis of CAT-AcK showed three major peaks (26898, 26974, and 27049 Da) corresponding to CAT-AcK (expected mass 26897 Da) and two different species with single or double β-ME (expected mass 26973 and 27049 Da respectively) (Fig. 3B right). The mass of CAT-AcK is 55 Da bigger than that of CAT because Asp112 is substituted by AcK. No deacetylated byproduct of CAT-AcK was detected. (Mass spectra before deconvolution are shown in Supplementary Figs. S3 and S4).

4. Concluding remarks

In this report, we demonstrated the evolution of AcKRS from *M. mazei* PylRS using a new selection system based on the killer gene *ccdB*. The selected MmAcKRS variants exhibited different mutation profile and showed higher in vivo suppression activities than the previously reported MbAcKRS variants [1,2]. We also determined the catalytic characteristics of the evolved AckRSs. To our knowledge, this is the first description of the kinetic properties of AcKRS enzymes. It is obvious, that the affinity of AcK for its evolved enzyme is very low, about 500–1000 times lower than the standard affinity of an amino acid for its cognate aminoacyl-tRNA synthetase [25]. A rare biochemical study of an azidonorleucine activating enzyme evolved from methionyl-tRNA synthetase showed it to be 2750-fold less active than the parent enzyme [26]. Thus, a similar drop in activity is likely in many of the currently laboratory-evolved 'orthogonal' synthetase enzymes. This then poses new challenges to enzyme engineering (with larger library sizes and possibly better selection schemes). The lower rate of aminoacyl-tRNA formation may explain the lower yields of recombinant proteins with non-natural amino acids than is expected for recombinant proteins containing only standard amino acids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.029.

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